Reversal of neuropathic pain in diabetes by targeting glycosylation of Ca_{V}3.2 T-type calcium channels

Peihan Orestes,¹ ³ Hari Prasad Osuru,¹ William E. McIntire,⁴ Megan O. Jacus,¹ Reza Salajegheh,¹ Miljen M. Jagodic,¹ WonJoo Choe,¹ ⁶ JeongHan Lee,¹ ⁷ Sang-Soo Lee,⁸ ⁹ Kirstin E. Rose,¹ Nathan Poiro,¹ Michael R. DiGruccio,¹ ⁵ Katiresan Krishnan,⁵ Douglass F. Covey,⁵ Jung-Ha Lee,⁸ ⁹ Paula Q. Barrett,⁴ Vesna Jevtovic-Todorovic¹ ² ³ and Slobodan M. Todorovic¹ ² ³

¹Dept. of Anesthesiology, ²Neuroscience, ³Neuroscience Graduate Program and ⁴Dept. of Pharmacology at the University of Virginia Health System, Charlottesville, VA; ⁵Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO; ⁶Department of Anesthesiology and Pain Medicine, InJe University, Ilsan Paik Hospital & College of Medicine, Goyang-City, Gyunggi-do, South Korea; ⁷Department of Anesthesiology and Pain Medicine, Busan Paik Hospital, InJe University, College of Medicine, Busan, South Korea; ⁸Department of Life Science and ⁹Interdisciplinary Program of Biotechnology, Sogang University, Shinsu-Dong, Seoul 121-742, South Korea

Corresponding Author: Slobodan M. Todorovic
Department of Anesthesiology, University of Virginia Health System, Mail Box 800710, Charlottesville, VA 22908-0710
Phone 434-924-2283, Fax 434-982-0019, Email:st9d@virginia.edu

Running Title: glycosylation of T-channels and nociception

Number of figures: 8
Number of supplemental figures: 0
Number of supplemental tables: 1
Number of pages: 28
Number of words in abstract: 196
Number of words in Main Text: 3971
Number of References: 27

Key words: low-threshold calcium channel, nociception, ob/ob mice, painful diabetic neuropathy, N-glycosylation
ABSTRACT

It has been established that CaV3.2 T-type voltage-gated calcium channels (T-channels) play a key role in the sensitized (hyperexcitable) state of nociceptive sensory neurons (nociceptors) in response to hyperglycemia associated with diabetes which in turn can be a basis for painful symptoms of peripheral diabetic neuropathy (PDN). Unfortunately, current treatment for painful PDN has been limited by non-specific systemic drugs with significant side effects or potential for abuse.

We studied in vitro and in vivo mechanisms of plasticity of CaV3.2 T-channel in a leptin-deficient (ob/ob) mouse model of PDN.

We demonstrate that post-translational glycosylation of specific extracellular asparagine residues in CaV3.2 channels accelerates current kinetics, increases current density and augments channel membrane expression. Importantly, de-glycosylation treatment with neuraminidase inhibits native T-currents in nociceptors and in so doing completely and selectively reverses hyperalgesia in diabetic ob/ob mice without altering baseline pain responses in healthy mice.

Our study describes a new mechanism for the regulation of CaV3.2 activity and suggests that modulating the glycosylation state of T-channels in nociceptors may provide a way to suppress peripheral sensitization. Understanding the details of this regulatory pathway could facilitate the development of novel specific therapies for the treatment of painful PDN.
INTRODUCTION

Despite significant advances in glucose monitoring and insulin therapy, people with diabetes remain hyperglycemic during significant portions of the day placing them at increased risk for the development of diabetic complications including PDN. One of the notable features of early PDN is the development of chronic neuropathic pain manifested as allodynia and hyperalgesia (1,2,3). Unfortunately, currently available therapies have limited efficacy and/or serious side-effects. For example, gabapentin and pregabalin can relieve symptoms of painful PDN; however, more than 50% of patients using these drugs experience side effects, most notably excessive sedation, which limits their clinical use (2). Although opioids and non-steroidal pain killers are also partially effective for treatment of chronic painful disorders, their long-term use is associated with side-effects like gastrointestinal bleeding, tolerance and addiction. Hence, further research to develop mechanism-specific novel pain therapies is warranted.

Recent studies have established the importance of the Ca\(V\)\(_{3.2}\) subtype of T-channels in controlling the excitability of peripheral nociceptors in dorsal root ganglia (DRG) and supporting peripheral pain processing in animal models of PDN (4). Despite these interesting findings, no pharmacological approach targeting these channels has provided a significant therapeutic benefit to these patients. This is in part because the mechanisms underlying DRG T-channel plasticity in chronic pain disorders, like PDN, remain unknown. Here, we hypothesize that post-translational modification of Ca\(V\)\(_{3.2}\) channels in nociceptors via glycosylation contributes to painful symptoms in an animal model of PDN.
RESEARCH DESIGN AND METHODS

**Animals:** Ethical approval was obtained for all experimental protocols from the University of Virginia Animal Care and Use Committee, Charlottesville, VA. All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* adopted by the U.S. National Institute of Health. Every effort was made to minimize animal suffering and the number of animals used. We used our standard procedure for testing mechanical and thermal sensitivity as we described elsewhere (5). Statistical comparisons were made using one and two-way repeated ANOVAs (paw and time post-injection) followed by Holm-Sidak multiple comparison with statistical significance accepted if \( p < 0.05 \). All drug injections were performed in a blinded manner.

ECN ((3β,5α,17β)-17-hydroxyestrane-3-carbonitrile) was dissolved in 15% β-cyclodextrin ([2-hydroxypropyl]-β-cyclodextrin) (Cyc) solution (Sigma-Aldrich) and 750 µl of solution containing ECN or vehicle alone was injected intraperitoneally (i.p.).

**Electrophysiological studies.** Patch-clamp recordings from acutely dissociated DRG neurons and HEK (human embryonic kidney)-293 cells were described in details in our previous publication (6). The external solution for voltage-clamp experiments in HEK-293 cell experiments contained (in mM), 152 TEA-Cl, 2-10 BaCl₂, and 10 HEPES, adjusted to pH 7.4 with TEA-OH. For voltage-clamp experiments in DRG cells we used 2 mM Ca\(^{2+}\) in external solution instead of Ba\(^{2+}\). The external solution for current-clamp experiments and recordings of voltage-gated sodium currents contained (in mM), 140 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4. The
internal solution for voltage-clamp experiments with DRG neurons contained (in mM) 135 TMA-OH, 40 HEPES, 10 EGTA, and 2 MgCl₂, adjusted to pH 7.2 with hydrogen fluoride (HF) (7). The internal solution for voltage-clamp experiments with HEK-293 cells contained (in mM), 110 Cs-MeSO₄ 14 creatine phosphate, 10 HEPES, 9 EGTA, 5 Mg-ATP, and 0.3 Tris-GTP, adjusted to pH 7.3 with CsOH. The internal solution for current-clamp experiments contained (in mM), 130 KCl, 40 HEPES, 5 MgCl₂, 2 Mg-ATP, 1 EGTA, and 0.1 Na₃GTP, adjusted to pH 7.3 with KOH.

Statistical comparisons were made using paired or unpaired t-tests where appropriate. All data are expressed as mean ± standard error of the mean (SEM); p values are reported only when statistically significant (<0.05).

**Biochemical studies.** Construction of Expression Vectors- cDNA encoding the human Cav3.2 gene was subcloned into the mammalian expression vector pDoubleTrouble (pDT) (8), resulting in hexahistidine and FLAG tags at the N-terminus of the channel.

Cell Culture and Transfection- HEK-293 cells were cultured in Dulbecco’s Modified Eagle Media containing 10% fetal bovine serum. Stable cell lines expressing the epitope tagged Cav3.2 channel were selected using the above media containing 500µg/ml G418. Alternatively, the pDT vector containing Cav3.2 was used to transiently transfect cells. Fifteen centimeter plates were transfected with 35 µg each of Cav3.2 DNA and Lipofectamine 2000 at a ratio of 1:1, and harvested at 48 hours.

*De-glycosylation in vitro-* Purified ₆HIS/FLAG Cav3.2 channel was separately incubated with PNGase F (New England Biolabs), Endoglycosidases F1, F2 and F3 (Sigma) for one hour.
at 37°C according to the manufacturers’ instructions. Reactions were terminated by the addition of sample buffer.

Methods for purification of CaV3.2 channels and SDS-PAGE and immunoblotting are described in Supplemental Information.

**Immunostaining and imaging.** Confocal images were acquired using a Zeiss 510UV confocal microscope and LSM Image Examiner software. Single plane images were taken using a 100X oil-immersion objective. To quantify images the colocalization plug-in for ImageJ (NIH) was used.

**Construction of CaV3.2 N-glycosylation mutants.** CaV3.2 N-glycosylation mutants (N192Q, N271Q, and N1466Q) were generated by mutating Asn192, Asn271, and Asn1466 residues of human CaV3.2 (GenBank accession no AF051946) into Gln residues using two-step PCR methods. The specific methods are described in Supplemental Information.

**RESULTS**

Recombinant CaV3.2 channels expressed heterologously in HEK-293 cells are typically cultured in a high-glucose medium to provide the cells’ energy supply. Interestingly, we determined that the glucose level in Invitrogen’s DMEM routinely used to grow these cells is 315 mg/dL (17.5 mmol/L) glucose, a value similar to blood glucose levels in diabetic ob/ob mice (5). We hypothesized that exposure of recombinant CaV3.2 channels to neuraminidase (NEU; at 1.5 U/ml for 1-3 h at 37°C), an enzyme that de-glycosylates proteins by removing sialic acid residues, would alter T-currents. We first characterized the effects of NEU on the current-voltage (I-V) relationship. We found that
depolarization to different test potentials ($V_t$) from a holding potential ($V_h$) of -90 mV resulted in similar normalized I-V relationships in control (Fig. 1A, top traces) and NEU-treated (Fig. 1A, bottom traces) cells (Figure 1B; untreated cells: ○, n=18 cells; NEU-treated cells: ●, n=13). Nevertheless, we found a visible slowing of macroscopic current kinetics of activation (as measured by 10-90% rise time) and inactivation (as measured by inactivation tau) after treatment with NEU in comparison to control, untreated cells (Fig. 1C and 1D) with a dramatic 2-fold slowing of the tau of inactivation. We also examined the effects of PNGase-F (PNG; at 20U/cc for 12 hours at 37°C) an enzyme that selectively cleaves N-glycosylated groups on proteins. Like NEU, PNG exposure slowed the kinetics of current activation and inactivation by two-fold (gray columns, Fig. 1E). Additionally, we found that treatment with NEU or PNG also reduced peak current density by about 40%, but that in combination, these agents were not more effective (Fig. 1F). Taken together, these data strongly suggest that NEU and PNG may share a common mechanism of modification of Cav3.2 channels.

Extracellular loops of ion channels may contain multiple potential motifs for glycosylation, which typically consist of an asparagine (Asn, N) residue separated from a serine (Ser, S) or threonine (Thr, T) residue by one amino acid (9). In Cav3.2 channels, conserved extracellular Asn residues in positions 192 (N192) and 271 (N271) of domain I, and 1466 (N1466) of domain III are excellent candidate sites for N-glycosylation (Fig. 2A). We hypothesized that Cav3.2 channels with mutated critical Asn residues reared in high glucose medium would have slower macroscopic kinetics of activation and inactivation than wild-type (WT) Cav3.2 channels. To test this hypothesis, we generated
single-point mutants of CaV3.2 channels in which one Asn residue was mutated into a glutamine (Q), namely N192Q, N1466Q and N271Q. Using transiently-transfected HEK-293 cells, we first compared the sensitivity of currents carried by N192Q mutant and WT CaV3.2 channels to nickel, a traditional CaV3.2 blocker. Traces depicted in Figure 2B show that 100 µM nickel applied in the external solution almost completely blocked inward currents carried by WT (top traces) and mutant channels (bottom traces). Hence, N192Q mutation did not alter a signature pharmacological property of CaV3.2 channels. Nevertheless, consistent with a functional role for putative N192 glycosylation in channel activity, macroscopic current kinetics of activation and inactivation of N192Q mutant channels (n=15) were significantly slower than those of WT channels (n=22; p<0.001; Figure 2C). Interestingly, current density (V_h -90 mV, V_t -30 mV) in N192Q channel mutant (17±5 pA/pF, n=13) was not different from WT CaV3.2 channel (15±3 pA/pF, n=7, p>0.05). In contrast to the N192Q channel mutant, currents were not evident in recordings of N271Q and N1466Q channel mutants (n ≥ 10, data not shown).

We next hypothesized that glycosylation state may regulate plasma membrane expression of CaV3.2 channels. To test this hypothesis, we constructed GFP-tagged WT CaV3.2 channels (EGFP-CaV3.2) and mutant channels containing disrupted putative glycosylation motifs, (EGFP-N192Q, EGFP-N271Q and EGFP-N1466Q), expressed them in HEK-293 cells and used confocal microscopy to quantify immunofluorescence. Figure 3 illustrates a representative experiment from each channel type (WT CaV3.2:Fig. 3A; mutant channels: Figs. 3B-D) showing red immunofluorescence for the structural membrane protein concanavalin A (left panels, Fig. 3A-D) and green immunofluorescence for the EGFP-tagged channel protein (middle panels, Fig 3A-D). As
indicated by the merging of red and green fluorescence (yellow color on right panels of Figs. 3A-D) we found that EGFP co-localized well with concanavalin A for WT (Fig. 3A) as well as N192Q (Fig. 3B) and N271Q (Fig. 3C) channel mutants consistent with predominant plasma membrane expression of these channel constructs. In contrast, the EGFP-N1466Q mutant channel was expressed predominantly in cytoplasmic organelles in agreement with lack of co-localization of the fluorescent signals (right panel of Fig. 3D). Average data from similar experiments were quantified blinded with arbitrary co-localization values and are summarized in Fig. 3E. Co-localization values were not significantly different for N192Q and N271Q mutants compared to WT CaV3.2 channels. By contrast, there is a large decrement in co-localization for N1466Q mutant: 28% of that of WT CaV3.2 channels (n = 4, p < 0.01), corroborating the lack of T-currents measured in our patch-clamp experiments in HEK cells expressing the N1466Q mutant.

Since two of our point mutations are located in repeat I of CaV3.2 channels (Fig. 2A), we generated N-terminus FLAG-tagged-CaV3.2 channels (6HIS/FLAGCaV3.2) to enable ensuing biochemical studies. We used the FLAG-tag to immunoprecipitate the 6HIS/FLAGCaV3.2 channel from HEK-293 cells grown in high glucose. Next, we treated the purified 6HIS/FLAGCaV3.2 channel with enzymes such as PNGase-F and endoglycosidases (Endo) F1, F2 and F3. De-glycosylation of proteins can result in a faster electrophoretic mobility, and thus a lower apparent molecular weight (MW). In the case of the full length CaV3.2 channel (about 260 kDa), very little change in electrophoretic mobility was observed after treatment with these enzymes (Fig. 4, top arrow) likely due to the large size of the protein. However, a small N-terminal fragment of the CaV3.2 channel recognized by the FLAG antibody shifted its electrophoretic mobility after treatment with
PNGase-F and Endo F1 consistent with a change in apparent MW, from 60 kDa to 50 kDa (Fig. 4, bottom arrow). In contrast, treatments of 6HIS/FLAG Ca\textsubscript{v}3.2 channel purified from HEK-293 cells with enzymes that are known to cleave complex carbohydrate molecules, such as Endo F2 and Endo F3 did not affect electrophoretic mobility of N-terminal fragment of Ca\textsubscript{v}3.2 (Fig. 4, bottom arrow). Thus, our data strongly suggest that the Ca\textsubscript{v}3.2 channel is glycosylated with a relatively simple sugar moiety within the first ~500 residues (50 kDa).

Next, we asked if glycosylation may modulate native Ca\textsubscript{v}3.2 currents in DRG nociceptors and may participate in painful PDN. Based on our recent studies using ob/ob mice, the up-regulation of T-currents in small DRG neurons coincided with significant hyperglycemia, morbid obesity, and the development of painful PDN in mice aged 10-16 weeks (5). Thus, we first compared the biophysical properties of T-currents in acutely dissociated small DRG cells from diabetic ob/ob and healthy WT mice at age 10-16 weeks. Representative traces of T-currents in DRG cells from control (Fig. 5A) and ob/ob mice (Fig. 5B) indicate marked enhancement of their amplitudes in ob/ob mice over the wide range of membrane potentials. We also found significant (up to two-fold) speeding of the kinetics of macroscopic current inactivation and activation in ob/ob mice (n = 20 cells, ●) compared to the WT group (n = 27 cells, ○) at all potentials tested (Figs. 5C and D, N ≥ 6 mice). In contrast, there was no apparent difference between the two groups in the voltage dependence of activation (Fig. 5E) or inactivation (Fig. 5F) of T-currents. Shown in Figure 6 are representative T-currents in DRG cells treated with NEU isolated from WT (Fig. 6A top traces) or ob/ob mice (Fig. 6A bottom traces). Bar graphs in Fig. 6
summarize average data from similar experiments. Interestingly, treatment with NEU (filled columns) did not significantly change T-current density in cells from WT mice, but effectively decreased T-current density in ob/ob group to WT levels (~17pA/pF, Fig. 6B). Furthermore, NEU significantly slowed both WT and ob/ob control (open columns) T-current kinetics to similar levels, tau of inactivation to ~105 ms and 10%-90% rise time of ~28 ms (Figs. 6C and D).

We also tested the possibility that NEU may affect other parameters besides T-currents in ob/ob mice using voltage- and current-clamp recordings from small DRG cells of diabetic ob/ob mice. In these experiments we blindly assigned saline-treated and NEU-treated dishes from the same preparations of DRG cells. Cells were first voltage-clamped and inward currents were evoked with a series of depolarizing pulses of 200 ms duration ($V_h = -90 \text{ mV, } V_t = -60 \text{ mV}$ through 0 mV). We found no significant difference in the peak amplitude of voltage-gated sodium currents in two groups: saline $230\pm28 \text{ pA/pF (n=25 cells)}$ and NEU $307\pm46 \text{ pA/pF (n=18 cells, p>0.05, N \geq 6 mice per group)}$. In ensuing current-clamp experiments in some of these cells we injected a series of incremental hyperpolarizing pulses and we measured passive membrane properties such as soma diameter, membrane capacitance, input resistance and resting membrane potential. Table S1 summarizes these data and indicates that NEU treatment did not have significant effects on any of these parameters (p>0.05).

We previously demonstrated that selective T-channel blocker ECN (10) at the dose of 25 mg/kg i.p. effectively reversed hyperalgesia in diabetic ob/ob mice and diabetic WT mice but had no effect on nociception in diabetic Cav3.2 knock-out (KO)
mice (5). However, the effects of NEU on pain thresholds in whole animals are not known. Hence, we examined whether NEU modifies in vivo sensitivity to mechanical and noxious thermal (heat) stimuli. We first measured baseline paw withdrawal responses (PWRs) in the presence of a mechanical stimulus elicited by von Frey filament (time point 0), then injected (arrowheads, Fig. 7) 10 µl of NEU or vehicle in the hind paws of WT (N = 5) or diabetic ob/ob mice (N = 10). We then measured PWRs in both paws at 30, 60 and 90 min after injection. As confirmed in Figs. 7A and 7B, ob/ob mice had increased baseline PWLs of about 60% as compared to their age-matched WT counterparts (Figs. 7C and D), suggestive of prominent mechanical hyperalgesia (5,11). Importantly, intraplantar (i.pl.) injection of 1.5 U/ml of NEU (■), the same concentration used in our in vitro experiments, completely reversed mechanical hyperalgesia in the injected (right) paws of ob/ob mice (Fig. 7A). In contrast, saline injections (○) did not cause any significant changes in PWRs (Fig. 7A). Interestingly, there was a transient hyperalgesic response to injections of NEU (■) in WT mice while saline injections (○) did not cause any alterations in baseline PWRs (Fig. 7C). PWLs in uninjected (left) paws of ob/ob mice (Fig. 7B) and WT mice (Fig 7D) remained stable throughout testing, indicating a lack of systemic effect. To test whether the anti-hyperalgesic effect of NEU in ob/ob mice is related to its effects on T-currents, we injected 25 mg/kg of ECN i.p. about 60 min before i.pl. injections of NEU (N = 8, Fig. 7E). As expected, the injections of ECN completely reversed mechanical hyperalgesia in ob/ob mice as evidenced by a notable decrease of mechanical PWRs in both paws measured 60 minutes after injections (#, p<0.001). Importantly, subsequent i.pl. injections of NEU did not further decrease PWRs in right paws. Unlike ECN the i.p. injections of Cyc (vehicle) did not change
baseline mechanical PWRs in ob/ob mice and subsequent local injections of NEU into right paws decreased significantly PWRs up to about 50% (Fig. 7F, ■). PWLs in uninjected (left) paws of the same ob/ob mice remained stable throughout testing, indicating a lack of systemic effect of NEU (Fig. 7F, ○).

Next we measured baseline paw withdrawal latency (PWL) in the presence of a radiant heat stimulus (time point 0), then injected (arrowheads, Fig. 8) 10 µl of NEU or vehicle i.pl. in 16-week old WT (N = 7) or ob/ob mice (N = 7). Ob/ob mice (Fig. 8A, top panel) had decreased baseline PWLs (4.0 ± 0.2 sec) for about 20% as compared to their age-matched WT counterparts (5.1 ± 0.3 sec, p<0.01) (Fig. 8A, bottom panel) indicating mild heat hyperalgesia (5). Importantly, i.pl. injection of 1.5 U/ml of NEU, produced a significant anti-hyperalgesia as evident by an increase in PWLs only in ob/ob mice at 30 min after injection (Fig. 8A, top panel) but not in WT mice (Fig. 8A, bottom panel). In control experiments, the same volume of saline (vehicle), had no effect on thermal PWLs in either WT or ob/ob mice (N ≥ 7, data not shown). As depicted on Fig. 8B, the i.p. injections of ECN had analgesic effects in ob/ob mice as evidenced by a notable increase of thermal PWLs in both paws (N = 4, p<0.001). Importantly, subsequent i.pl. injections of NEU did not further prolong PWLs in right paws.

DISCUSSION
It has been established that T-channels can contribute to the hyperexcitability of sensory neurons manifested by hyperalgesia and allodynia, two frequent symptoms of chronic neuropathic pain (4,12). Several studies have validated that plasticity of T-channels is implicated in hyperalgesia and allodynia in animal models of PDN (5,12-17). Taken
together, these studies identify an important pro-nociceptive role of Cav3.2 T-channels in neuropathic pain in animal models of Type 1 and Type 2 diabetes mellitus. However, molecular mechanisms for the alteration of Cav3.2 channels in DRG cells from diabetic animals have not been previously described. Indeed, herein we provide evidence for the first time that targeting glycosylation states of T-channels may be a promising new treatment for painful PDN. This conclusion is based on several observations from our study. First, we show that macroscopic current activation and inactivation kinetics as well as current density are drastically reduced when recombinant human Cav3.2 channels expressed in HEK-293 cells reared in hyperglycemic cell culture medium are exposed to NEU and PNG. Second, the effects of NEU are more prominent in DRG cells from diabetic ob/ob mice than healthy WT mice. Thirdly, NEU in vivo completely reversed thermal and mechanical hyperalgesia in diabetic ob/ob mice whereas it was completely ineffective in age-matched healthy WT mice. The fact that NEU modified DRG T-current kinetics from healthy WT mice but to a lesser extent than in ob/ob mice suggests that the level of T-channel glycosylation is an important physiological mechanism that fine tunes the activity of these channels in pain pathways. However, it appears that this process is maladaptive and leads to cellular hyper-excitability and consequently hyperalgesia in diseases like PDN. Hence, glycosylation of Cav3.2 channels is an important mechanism of sensitization of peripheral nociceptors that could be exploited for novel pain therapies.

Our molecular studies identify conserved extracellular asparagine residues most notably N192 and N1466, as important regulators of Cav3.2 current kinetics and channel membrane expression, respectively. This is supported by our patch-clamp recordings that demonstrated slower current kinetics in N192Q Cav3.2 mutant with apparently normal
membrane expression. In contrast, we could not consistently record T-currents in HEK-293 cells transfected with N1466Q Ca\(_{\text{V}}\)3.2 mutant and our imaging studies with the EGFP-tagged mutant showed minimal membrane expression. Thus, different glycosylation sites in Ca\(_{\text{V}}\)3.2 channels may have distinct functional roles. Surprisingly, we could not record T-currents from N271Q Cav3.2 channels despite apparently normal membrane expression. It remains possible that N271Q channels trafficked to the membrane are nonfunctional. During the review of our study another \textit{in vitro} study using recombinant human Ca\(_{\text{V}}\)3.2 channels also examined the effect of glycosylation on T-current kinetics and surface membrane expression (18). Surprisingly, they found that treatments with PNG but not NEU decreased Ca\(_{\text{V}}\)3.2 current density and slowed kinetics of channel inactivation. Furthermore, their work suggests that asparagine N192 serves as a regulator of channel membrane expression and asparaginesN1466 as a regulator of channel kinetics. It is possible that specific conditions of enzymatic de-glycosylation and/or different levels of glucose in cell culture could have contributed to the different findings between the studies. However, regardless of observed differences, our study directly demonstrates that Ca\(_{\text{V}}\)3.2 -channels are indeed glycosylated within domain I of the channel protein, and for the first time reveals the prominent effects of NEU on native T-currents in DRG cells and on pain perception \textit{in vivo} using an animal model of PDN.

Several other \textit{in vitro} studies have reported that glycosylation may modulate properties of other voltage-gated ion channels. For example, in embryonic DRG neurons, NEU affected steady-state inactivation of voltage-gated sodium channels (19). While future biophysical studies may reveal fine details of the effects of glycosylation upon Ca\(_{\text{V}}\)3.2 current kinetics, it is reasonable to propose that increased current density and
increased kinetics of CaV3.2 current activation alone may contribute to the hyperexcitable state of DRG cells under hyperglycemic conditions. Similar to our study, Tayrrell and colleagues did not find any effects of NEU on voltage-gated sodium channels in small DRG cells from adult animals. Future extensive electrophysiological studies could be expanded to involve examination of other voltage-gated channels that are crucial for the control of cellular excitability of DRG cells from diabetic animals that might also be modulated by glycosylation (20-22).

Previous in situ hybridization studies (23) and electrophysiological studies using KO mice (6,24) have established that the CaV3.2 is the most prevalent isoform of the Cav3.0 family in small DRG cells. Thus, we have focused our investigation on the effects of glycosylation on this particular isoform and on the peripheral sensitization of pain responses in PDN. Our molecular studies have found that extracellular asparagine residues N192 and N1466 are likely putative substrates for glycosylation that alter T-channel membrane expression and current kinetics. Since these asparagine residues are conserved across all T-channel isoforms it is likely that glycosylation may similarly modulate the other two T-channel isoforms, namely CaV3.1 and CaV3.3. Interestingly, all three isoforms of T-type channels are expressed in dorsal horn neurons of spinal cord (23) and recent studies have shown that they all may support spinal nociceptive processing in different animal models of neuropathic pain (12,25,26). Thus, simultaneous glycosylation of multiple T-channel isoforms in spinal dorsal horn neurons may contribute to their hyper-excitability, which in turn may influence central sensitization of pain responses that is implicated in many pain disorders (27).
Overall, the results presented here fundamentally advance our understanding of the mechanisms of glycosylation underlying the post-translational modification of Cav3.2 T-channels that has an important function in supporting peripheral nociceptive signaling. Our results strongly suggest that the manipulation of glycosylation states of peripheral nociceptors could be useful for the development of novel therapies for the treatment of painful PDN. This method may have an advantage over direct blockers of T-channels to suppress pain because NEU and related agents will correct the pathology of diabetes at its source, rather than ameliorating the problem through separate pathways that also may be a source of unintended side effects. Our goal is to provide novel therapeutic modalities that would not only alleviate neuropathic pain in patients with diabetes but, even more importantly, halt its progression without causing dangerous systemic side effects or creating the potential for drug abuse.

ACKNOWLEDGEMENTS

Our research is supported by American Diabetes Association 7-09-BS-190 (to S.M.T.), Dr. Harold Carron Endowment fund, Priority Research Centers Program through the NRF of Korea (2012-0006690 to JH Lee), NIH grant -7 HL 036977 (to P.Q.B.) and funds from the Department of InJe University. No potential conflicts of interest relevant to this article were reported.

Parts of this study were presented at the 39th Annual Society for Neuroscience Meeting, Chicago Illinois, 17-21 October 2009; 40th Annual Society for Neuroscience Meeting, San Diego California, 13-17 November 2010; 2-nd Conference on Calcium Channel Research, Placencia, Belize March 28- April 3 2010; and 3-rd Conference on Calcium Channel Research, Krabi, Thailand, March 24-29, 2013.
We thank Mr. Damir Bojadzic for technical assistance and Dr. Jan Redick and Dr. Stacey Guillot of the Advanced Microscopy Core Facility (UVA) for help with imaging studies. S.M.T. is a guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Author Contributions: P.O. researched data, wrote the manuscript and contributed to discussion, H.P.O. researched data, W.E.M. researched data and contributed and reviewed/edited manuscript, M.O.J. researched data, R.S. researched data, M.M.J. researched data, W.J.C. researched data, J.H.L. researched data, S-S. L. researched data, K.E.R researched data, N.P. researched data, M.R.D. researched data, K.K. researched data, D.F.C. researched data, contributed to discussion, reviewed/edited the manuscript, J-H.L. contributed to discussion, reviewed/edited the manuscript, P.Q.B. researched data, wrote manuscript, reviewed/edited the manuscript, V.J-T. researched data, contributed to discussion, wrote manuscript, reviewed/edited the manuscript, S.M.T. researched data, wrote the manuscript, contributed to discussion, reviewed/edited the manuscript.

REFERENCES


10) Todorovic SM, Prakriya M, Nakashima YM, Nilsson KR, Han M, Zorumski CF, Covey, DF, Lingle CJ. Enantioselective blockade of t-type Ca\textsuperscript{2+} current in adult rat sensory neurons by a steroid that lacks \gamma-aminobutyric acid-modulatory activity. Mol Pharmacol 1998; 54: 918-927.


18) Weis N, Black SAG, Bladen C, Chen L, Zamponi GW. Surface expression and function of CaV3.2 T-type calcium channels are controlled by asparagines-linked glycosylation. Pflugers Arch-Euro J Physiol 2013; Epub Ahead of Print


FIGURE LEGENDS

Figure 1. Neuraminidase and PNGase-F modulate recombinant human CaV3.2 channels.

A. Traces represent families of T-currents evoked in representative HEK-293 cells in control conditions (top panel) and after incubation of 1.5 U/ml of NEU at 37\(^{0}\) C for 3 hours (lower panel) by voltage steps from -90 mV (V\(_h\)) to V\(_t\) from -80 through -20 mV in 5-mV increments. Bars indicate calibration.

B. Average normalized I-V curves are shown in HEK-293 cells in control conditions (open symbols, n= 18) and after incubations of NEU (filled symbols, n = 13). C,D. We measured time-dependent activation (10%-90% rise time, panel C) and inactivation \(\tau\) (single exponential fit of decaying portion of the current waveforms, panel D) from I-V curves in HEK-293 cells (shown in panel B of this figure) over the range of test potentials from -50 mV to 10 mV. There are differences in up to two-fold slower times between the control (open symbols) and NEU groups (black filled symbols) at each tested potential. * denotes significance of p<0.05.
E. Bars represent 10-90 % current activation rise times (on the left) and current inactivation \(\tau\) \((V_h = -90 \text{ mV}, V_t = -30 \text{ mV})\) measured in control cells (open bars, \(n=13\)) and cells incubated with PNGase-F (PNG) for 20 U/ml at 37\(^\circ\)C for 12 hours (gray bars, \(n=7\)). Note that PNG-treated cells had slower activation and inactivation kinetics. * denotes significance of \(p<0.01\)

F. Bar graphs depicts peak current density \((V_h = -90 \text{ mV}, V_t = -30 \text{ mV})\) measured in multiple HEK-293 cells in control conditions (open bar, \(n=20\)) and cells after incubation of NEU alone (black filled bar, \(n=18\)), PNG alone (light gray bar, \(n=7\)) and combined PNG and NEU (dark gray bar, \(n=11\)). Note that all 3 treatments significantly decreased peak current density when compared to control cells: control 95±10 (open bar, \(n=20\)), NEU 65±8 (black filled bar, \(n=18\), \(p<0.01\)), PNG 59±2 (light gray bar, \(n=7\), \(p<0.01\)) and NEU+PNG 53±5 (dark gray bar, \(n=11\), \(p<0.01\)). * denotes significance of \(p<0.05\)

Vertical bars on all panels of this figure represent SEM from multiple determinations.

**Figure 2. Molecular mechanisms of glycosylation of Ca\(_{\text{v3.2}}\) channels.**

A. Schematic diagram of Ca\(_{\text{v3.2}}\) showing the position of conserved putative N-glycosylation sites in the extracellular face of the channel in domains I and III. Designated asparagine residues (in red bold fonts) were mutated to alanine residues.

B. Representative traces in gray show nickel inhibition of T-current (black traces) in HEK-293 cell transiently transfected with WT Ca\(_{\text{v3.2}}\) (top) and N192Q Ca\(_{\text{v3.2}}\) channels (bottom). In both experiments, 100 \(\mu\)M NiCl\(_2\) was applied in the bath. On average nickel blocked 97 ± 2% of inward currents of N192Q Ca\(_{\text{v3.2}}\) (\(n=5\)) and WT Ca\(_{\text{v3.2}}\) channels (\(n=6\)). Bars indicate calibration.
C. Bar graph represents the average effect of N192Q Ca\textsubscript{v}3.2 mutation (black bars) when compared to WT Ca\textsubscript{v}3.2 (open bars) T-current kinetics ($V_h = -90$ mV, $V_t = -30$ mV) in HEK-293 cells. In average N192Q mutant has slower 10-90% rise times by about 60% (8.2 ± 0.8 sec) when compared to WT Ca\textsubscript{v}3.2 currents (5.1 ± 0.2 sec). Similarly, in average N192Q mutant has slower inactivation $\tau$ values by about 50% (31.4 ± 2.2 sec) when compared to WT Ca\textsubscript{v}3.2 currents (21.8 ± 0.6 sec). Vertical lines are ± SEM of multiple determinations. Number of cells in each experiment is indicated in parentheses. * denotes significance of $p<0.001$

**Figure 3. Altered membrane expression of putative glycosylation sites in Ca\textsubscript{v}3.2 channels.**

**A.** Representative confocal images on the left show HEK-293 cells transiently transfected with WT Ca\textsubscript{v}3.2 channels where concanavalin A immunofluorescence was represented in red color. Middle panel shows green immunofluorescence representing EGFP tagged Ca\textsubscript{v}3.2 transfection in the same cells. Right panel shows merged images where overlay is presented in yellow color.  

**B.** Representative confocal images on the left show HEK-293 cells transiently transfected with N192Q Ca\textsubscript{v}3.2 channels where concanavalin A immunofluorescence was represented in red color. Middle panel shows green immunofluorescence representing EGFP tagged N192Q Ca\textsubscript{v}3.2 transfection in the same cells. Right panel shows merged images where overlay is presented in yellow color.  

**C.** Representative confocal images on the left show HEK-293 cells transiently transfected with N271Q Ca\textsubscript{v}3.2 channels where concanavalin A immunofluorescence was represented in red color. Middle panel shows green immunofluorescence representing
EGFP tagged N271Q Ca\textsubscript{V}3.2 transfection in the same cells. Right panel shows merged images where overlay is presented in yellow color. D. Representative confocal images on the left show HEK-293 cells transiently transfected with N1466Q Ca\textsubscript{V}3.2 channels where concanavalin A immunofluorescence was represented in red color. Middle panel shows green immunofluorescence representing EGFP tagged N1466Q Ca\textsubscript{V}3.2 transfection in the same cells. Right panel shows merged images where overlay is presented in yellow color. Note that EGFP and concanavalin A show very little overlap in their sub-cellular distributions. E. Bar graphs represent average values from multiple experiments as those depicted on panels A-D of this figure. Colocalization values for EGFP and concanalvalin epifluorescence were quantified and compared between mutants and WT Ca\textsubscript{V}3.2 channels. Only N1466Q mutants (dark gray column, 9.9 ± 3.7, n=4) displayed about 73% decreased in colocalization value when compared to WT channels (black column, 36.2 ± 3.8, n=4). In contrast, colocalization values of N192Q (open column, n = 4) and N271Q mutants (light gray bar, n=3) were not significantly different from WT Ca\textsubscript{V}3.2. * indicates a statistically significant difference from Ca\textsubscript{V}3.2 WT channels (p < 0.01) and NS (not significant) indicates p > 0.05 when compared to Ca\textsubscript{V}3.2 WT channels. Calibration bars are marked on all panels of this figure.

**Figure 4. Biochemical evidence of glycosylation of Ca\textsubscript{V}3.2 channels.**

Immunoblotting with FLAG antibody reveals a shift in apparent molecular weight of N-terminal fragment of Ca\textsubscript{V}3.2 channel (bottom arrow), but not full channel (top arrow). Note that treatment with PNGase-F and Endo F1 but not Endo F2 and Endo F3 caused an obvious change in mobility of FLAG-labeled N-terminal fragment of Ca\textsubscript{V}3.2 from about
60 to 50 KDa. Using mass spectrometry we confirmed that the de-glycosylated N-terminal fragment of Ca\textsubscript{V}3.2 channels recognized by FLAG antibodies contains most of the repeat I of Ca\textsubscript{V}3.2 channel (data not shown).

**Figure 5. Alterations of macroscopic T-current kinetics in acutely dissociated small DRG cells from diabetic ob/ob mice.**

The data show original T-current traces ($V_h$ -90 mV, $V_t$ -80 mV through -30 mV) from representative DRG cells from a healthy WT mouse (Panel A), and a diabetic ob/ob mouse (Panel B). The averaged data show marked acceleration in T-current inactivation (Panel C) and activation (Panel D) kinetics in ob/ob mice (black filled symbols) when compared to age-matched WT mice (open symbols). Data are averages of multiple cells (WT n = 27, ob/ob n = 20) ± S.E.M. *, p<0.05. Solid lines on panels C and D are single exponential fits to experimental data points. E. Normalized peak T-current activation curves from similar experiments shown in the panels A and B of this figure. Open symbols represent the WT mice and black filled symbols represent the cells from ob/ob mice. Number of cells is indicated in the parentheses. Solid black lines are fitted using equation #1, giving half-maximal activation ($V_{50}$), which occurred at -46.6 ± 0.6 mV with a $k$ of 6.3 ± 0.5 mV in WT mice. Similarly $V_{50}$ was -47.6 ± 0.7 mV with a $k$ of 7.4 ± 0.6 mV in the DRG cells from ob/ob mice. F. Normalized peak T-current steady-state inactivation curves. T-currents are evoked by test steps to -30 mV after 3.5-sec prepulses to potentials ranging from -110 mV to -45 mV in 5-mV increments. Open symbols represent the WT mice and black filled symbols represent the cells from ob/ob mice. Number of cells is indicated in the parentheses. Solid black lines are fitted using equation
#2, giving half-maximal inactivation ($V_{50}$), which occurred at $-75.0 \pm 0.4$ mV with a $k$ of $8.9 \pm 0.4$ mV in WT mice. Similarly $V_{50}$ was $-75.8 \pm 0.4$ mV with a $k$ of $8.2 \pm 0.3$ mV in the DRG cells from ob/ob mice.

The voltage dependencies of activation and steady-state inactivation were described with single Boltzmann distributions of the following forms:

**Activation:**
$$I(V) = \frac{I_{\text{max}}}{1 + \exp \left[ \frac{(V-V_{50})}{k} \right]}$$  \hspace{1cm} (1)

**Inactivation:**
$$I(V) = \frac{I_{\text{max}}}{1 + \exp \left[ \frac{(V-V_{50})}{k} \right]}$$  \hspace{1cm} (2)

where $I_{\text{max}}$ is the maximal activatable current, $V_{50}$ is the voltage where half the current is activated or inactivated, and $k$ is the voltage-dependence (slope) of the distribution.

**Figure 6. Neuraminidase treatment in vitro reversed kinetic alterations and normalized T-current density in small DRG cells from diabetic ob/ob mice.**

**A.** Traces represent families of T-currents evoked in representative DRG cells in a WT mouse (top panel) and a diabetic ob/ob mouse after incubation of 1.5 U/ml of NEU at 37° C for 3 hours (lower panel) by voltage steps from $V_h = -90$ mV to $V_t$ from -80 through -25 mV in 5-mV increments. Bars indicate calibration. **B.** Bar graphs with the averaged data show that NEU treatments completely reversed DRG T-current density ($V_h = -90$ mV, $V_t = -30$ mV) (Panel B), T-current inactivation measured by inactivation $\tau_s$ (Panel C) and activation kinetics measured by 10-90% rise time (Panel D) in ob/ob mice (right panels) when compared to healthy WT mice (left panels). Controls (open symbols) were compared to post-neuraminidase (NEU) treatments (filled symbols). DRG cells were freshly dissociated as noted in Fig. 5. Recordings were performed at room temperature while NEU was incubated for 1-3 hours at 37° C. Control cells were treated with saline.
Data are averages of multiple cells as indicated in parenthesis ± S.E.M.). *, p<0.001; n.s. not significant, p>0.05

**Figure 7. Neuraminidase treatment *in vivo* reversed mechanical hyperalgesia in diabetic ob/ob mice.**

A. The graph shows average data points indicating that i.pl. injections of NEU (solid black squares) but not saline (open circles) into right paws completely reversed mechanical hyperalgesia in diabetic ob/ob mice. Arrow indicates time point of i.pl. injections. * indicates significant change of PWRs with p < 0.05 when compared to baseline PWR prior to i.pl. injections. 

B. The graph shows average data points indicating that i.pl. injections of NEU (solid black squares) and saline (open circles) into right paws did not affect PWRs in the left paws of diabetic ob/ob mice. Arrow indicates time point of i.pl. injections.

C. The graph shows average data points indicating that i.pl. injections of NEU (solid black squares) but not saline (open circles) into right paws caused transient hyperalgesia in healthy WT mice at the time point of 30 minutes. Arrow indicates time point of i.pl. injections. * indicates significant change of PWRs with p < 0.001 when compared to baseline PWR prior to i.pl. injections. 

D. The graph shows average data points indicating that i.pl. injections of NEU (solid black squares) and saline (open circles) into right paws did not affect PWRs in the left paws of WT mice. Arrow indicates time point of i.pl. injections.

E. The graph with averaged data from 8 experiments show that i.p. injections (thin arrow) of selective T-channel blocker ECN at 25 mg/kg completely reversed mechanical hyperalgesia in diabetic ob/ob mice as evidenced by decreased PWRs in both right (solid black symbols) and left (open bars) paws (#, p<0.001). Subsequent i.pl. injections (thick arrow) of 1.5 U/ml of NEU in the
same animals did not significantly influence new baseline values of mechanical PWRs at time point of 90 minutes, but it significantly increased PWRs at time points of 120, 150 and 180 minutes (*, p < 0.05). F. The graph with averaged data from 8 experiments show that i.p. injections (thin arrow) of vehicle used to dissolve ECN (Cyc) did not affect mechanical hyperalgesia in diabetic ob/ob mice as evidenced by stable PWRs in both right (solid black symbols) and left (open bars) paws at time point of 60 minutes. Subsequent i.pl. injections (thick arrow) of 1.5 U/ml of NEU in the same animals effectively reversed diabetic hyperalgesia by significantly decreasing PWRs at time points of 120, 150 and 180 minutes (*, p < 0.001).

Figure 8. Neuraminidase treatment in vivo reversed thermal hyperalgesia in diabetic ob/ob mice.

A. The bar graphs with averaged data show that i.pl. injections of NEU completely reversed thermal hyperalgesia in diabetic ob/ob mice (top) while same treatment was ineffective in WT mice (bottom). Neuraminidase (NEU) was injected into right (R) paws (filled bars) while un-injected left (L) paws served as controls (open bars). Paw withdrawal latencies (PWLs) were determined in mice before (time point 0) and 10 and 30 minutes after injections of 10 µL of NEU (arrowheads) into hind paws. Data are averages of 7 experiments ± S.E.M. Symbols indicating significance of NEU treatments are as follows: *, p<0.01 for R vs. L paws at same time points; †,p<0.01 for data points at 30 minutes after NEU injections vs. 0 minutes. B. The bar graphs with averaged data show that i.p. injections of selective T-channel blocker ECN at 25 mg/kg completely reversed thermal hyperalgesia in diabetic ob/ob mice as evidenced by elevated PWLs in
both right (black bars) and left (open bars) paws (p<0.001). Subsequent intraplantar injections of 1.5 U/ml of NEU in the same animals did not significantly alter new baseline values of thermal PWLs (n.s., p>0.05, n=4). Arrows indicate times of injections of ECN and NEU.
Neuraminidase and PNGase-F modulate recombinant human CaV3.2 channels.
Molecular mechanisms of glycosylation of CaV3.2 channels.
221x273mm (300 x 300 DPI)
Figure 3

Concanavalin A  EGFP  Overlay

HEK293  Cav3.2
WT
N192Q
N271Q
N1466Q

Altered membrane expression of putative glycosylation sites in Cav3.2 channels.

187x194mm (300 x 300 DPI)
Biochemical evidence of glycosylation of CaV3.2 channels.

118x156mm (300 x 300 DPI)
Alterations of macroscopic T-current kinetics in acutely dissociated small DRG cells from diabetic ob/ob mice.
Neuraminidase treatment in vitro reversed kinetic alterations and normalized T-current density in small DRG cells from diabetic ob/ob mice.
Neuraminidase treatment in vivo reversed mechanical hyperalgesia in diabetic ob/ob mice.

180x237mm (300 x 300 DPI)
Neuraminidase treatment in vivo reversed thermal hyperalgesia in diabetic ob/ob mice.

149x249mm (300 x 300 DPI)
Construction of Ca$_{3.2}$ N-glycosylation mutants

Ca$_{3.2}$ N-glycosylation mutants (N192Q, N271Q, and N1466Q) were generated by mutating Asn192, Asn271, and Asn1466 residues of human Ca$_{3.2}$ (GenBank accession no AF051946) into Gln residues using two-step PCR methods. The specific methods are as follows.

**N192Q:** The forward and reverse primers to amplify the upper fragments covering 215 to 585 (nucleotide number of Ca$_{3.2}$) are 5’-ACCCGGCCTTGGCGGCA-3’ and 5’-GAGGCTCACCTGGTGTCGGTCCAACGAGTA-3’. The forward and reverse primers to amplify the lower fragments covering 565 to 1147 are 5’-GACGGACACCAGTGAGCCTCTCGGCTATC-3’ and 5’-TGATGTCGACCCAGCCTCCAGCGT-3’. The upper and lower fragments were overlapped and extended by second-step PCR. N192Q pcDNA3 was constructed by ligating SfiI (227)-BspEl (2560) PCR fragment and BspEl (2560, Ca$_{3.2}$)-Agel (3844, Ca$_{3.2}$) fragment into Ca$_{3.2}$ pcDNA3 opened with SfiI (227, Ca$_{3.2}$) and Agel (3844, Ca$_{3.2}$).

**N271Q:** The forward and reverse primers to amplify the upper fragments covering 215 to 823 (nucleotide number of Ca$_{3.2}$) were 5’-GCGGCCACGGTCTTCTTCTG-3’ and 5’-GAAGGTCAGCTGGTTGTTCCTGACAAAGGC-3’. The forward and reverse primers to amplify the lower fragments covering 806 to 1147 were 5’-AACAACCAGCTCTCTTCTGCGGCCG-3’ and 5’-TGATGTCGACCCAGCCTCCAGCGT-3’. The upper and lower fragments were overlapped and extended by second-step PCR. N271Q pcDNA3 was constructed by ligating SfiI (227)-BspEl (2560) PCR fragment and BspEl (2560, Ca$_{3.2}$)-Agel (3844, Ca$_{3.2}$) fragment into Ca$_{3.2}$ pcDNA3 opened with SfiI (227, Ca$_{3.2}$) and Agel (3844, Ca$_{3.2}$).

**N1466Q:** The forward and reverse primers to amplify the upper fragments covering 3840 to 4407 are 5’-AGAACCAGTTCCGCGTCT-3’ and 5’-GGTGGAGATCTGCTGGTGCAGGGCCCT-3’. The forward and reverse primers to amplify the lower fragments covering 4390 to 6537 are 5’-ACCAGGCAGATCTCCACCAAGCCACAG-3’ and 5’-AGAGCGGGCTCGGCTCA-3’. The upper and lower fragments were overlapped and
extended by second-step PCR. N1466Q pcDNA3 was constructed by ligating AgeI (3844)-Bsu36I (6520) PCR fragment into Ca3.2 pcDNA3 opened with AgeI (3844, Ca3.2) and Bsu36I (6520, Ca3.2).

EGFP-Ca3.2, EGFP-N192Q, EGFP-N271Q, and EGFP-N1466Q: For the green fluorescence experiments, we tagged EGFP (enhanced green fluorescence protein) cDNA to the amino termini of wild-type Ca3.2 and N-glycosylation mutants by ligating EGFP (ClaI-NotI) and NotI (263, Ca3.2)-BspEI (2560, Ca3.2) fragment into plasmids (Ca3.2, N192Q, N271Q, and N1466Q in pcDNA3) opened with ClaI (5'-polylinker) and BspEI (2560, Ca3.2).

**Purification of Cav3.2 Channel**- Stable HEK-293 cells expressing the 6HIS/FLAG Cav3.2 channel were harvested by washing with 10 mls of 37°C PBS, followed by incubation with PBS containing 5 mM EDTA for several minutes. Cells were collected by centrifugation at 900 rpm for 10 minutes, washed with ice cold PBS, resuspended in buffer containing 20 mM HEPES, pH 7.4, 1 mM EGTA, and protease inhibitors, and flash frozen. Cells were lysed with a 21 g needle, and membranes collected by centrifugation at 35,000 RPM for 45 minutes in a 45 Ti rotor at 4°C. Membranes were resuspended in 4 mls of buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% CHAPS and protease inhibitors using an 18 g needle, and allowed to rotate end over end for one hour at 4°C; extracts were clarified by high speed centrifugation as noted above, diluted tenfold with 20 mM HEPES, pH 7.4, 150 mM NaCl and protease inhibitors, and incubated with FLAG M2 beads overnight at 4°C. FLAG beads were collected in a five ml column and washed with five 1 ml volumes of buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% n-dodecyl beta-D-maltoside (DDM) and protease inhibitors; the column was then brought to room temperature, and 100 µl of the previously mentioned buffer containing 0.5 mg/ml FLAG peptide (also at room temperature) was added to the column and collected. The column was then capped, and a second 100 µl was added and
let incubate at room temperature for 15 minutes; after which time the volume was collected, and subsequent 100 µl volumes were added to elute the remainder of the 6HIS/FLAG Cav3.2 channel.

**SDS-PAGE and Immunoblotting** - Samples were prepared for separation by SDS-PAGE by incubation with sample buffer at room temperature for 1-2 hours; samples were not boiled. Protein separations were achieved either using a 4-15% precast gels (BioRad) or 12% mini-gels. Immunoblots from 4-15% precast gels were transferred overnight at 30 volts onto PVDF membrane at 4°C; alternatively, immunoblots from 12% mini-gels were transferred for one hour at 100 volts onto nitrocellulose at 4°C. Membranes were probed with monoclonal M2 FLAG antibody (Sigma) at a 1:1000 dilution. Immunoreactivity was detected with horseradish peroxidase coupled secondary antibody in conjunction with enhanced chemiluminescence. Polyacrylamide gels used for generation of samples for mass spectrometric analysis were prepared by 0.22 µM filtration of the separating and stacking solutions, as well as the running buffer; this step is important for removal of common protein contaminants, such as keratin, that can obscure the detection of sample proteins. Gels were stained in a 0.1% Coomassie Brilliant blue solution of 45:45:10 methanol:water:acetic acid, followed by destaining in a 45:45:10 methanol:water:acetic acid solution. Once protein bands were adequately visualized, gels were stored in a 10% acetic acid solution.
<table>
<thead>
<tr>
<th></th>
<th>Ob/Ob Control (n=7) mean ± SEM</th>
<th>Ob/Ob NEU (n=8) mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soma diameter (µm)</td>
<td>32.4 ± 1.0</td>
<td>30.5 ± 1.0</td>
</tr>
<tr>
<td>Capacitance (pF)</td>
<td>36.4 ± 4.5</td>
<td>27.8 ± 5.2</td>
</tr>
<tr>
<td>Resting membrane</td>
<td>-51.5 ± 4.5</td>
<td>-50.5 ± 3.9</td>
</tr>
<tr>
<td>potential (mV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Input Resistance (MΩ)</td>
<td>160.7 ± 30.8</td>
<td>176.4 ± 26.2</td>
</tr>
</tbody>
</table>

**Table 1.** Neuraminidase (NEU) does not have significant effects on passive membrane properties of acutely dissociated smaller DRG cells from Ob/Ob mice. Control cells (n=7) were treated with saline and experimental group (n=8) with 1.5 U/cc of NEU for 3 hours.